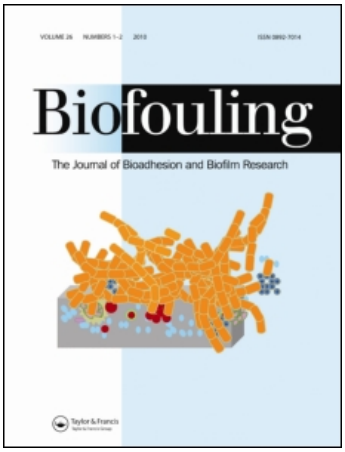

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Phage control of dual species biofilms of *Pseudomonas fluorescens* and *Staphylococcus lentus*

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Despite the recent enthusiasm for using bacteriophages as bacterial control agents, there are only limited studies concerning phage interaction with their respective hosts residing in mixed biofilm consortia and especially in biofilms where the host species is a minor constituent. In the present work, a study was made of mono and dual species biofilms formed by *Pseudomonas fluorescens* (Gram-negative) and/or *Staphylococcus lentus* (Gram-positive) and their fate after infection with phages. The dual species biofilms consisted predominantly of *S. lentus*. The exposure of these biofilms to a cocktail containing both *P. fluorescens* and *S. lentus* phages effectively killed and removed the hosts from the substratum. Additionally, this cocktail approach also controlled the hosts released from the biofilms to the planktonic phase. The ability of phages to control a host population present in minority in the mixed species biofilm was also assessed. For this objective, the biofilms were challenged only with phage ϕIBB-PF7A, specific for *P. fluorescens* and the results obtained were to some extent unpredicted. First, ϕIBB-PF7A readily reached the target host and caused a significant population decrease. Secondly, and surprisingly, this phage was also capable of causing partial damage to the biofilms leading to the release of the non-susceptible host (*S. lentus*) from the dual species biofilms to the planktonic phase. The efficiency of phage treatment of biofilms was to some extent dependent on the number of cells present and also conditioned by the infection strategy (dynamic or static) utilized in the infection of the biofilms. Nevertheless, in most circumstances phages were well capable of controlling their target hosts.

Keywords: *Pseudomonas fluorescens*; *Staphylococcus lentus*; biofilm; bacteriophage; control

Introduction

Microorganisms living attached to inert surfaces in multicellular consortia are known as biofilms. In this sessile lifestyle, the microorganisms are found embedded in a matrix where extracellular polymeric substances (EPS) are considered the primary matrix material (Flemming et al. 2000). Besides the microorganisms present and their physiological state, biofilm structure is influenced by physical factors such as availability of nutrients and oxygen, and the flow velocity of the surrounding liquid, and also by the surface on which the biofilm is formed (Sutherland 2001).

The susceptibility of biofilms to antimicrobial agents is clearly influenced by the biofilm mode of growth of the bacteria, especially in interspecies biofilms where several interactions occur between the different species present. This decreases the efficacy of the antimicrobial agents and leads to serious problems particularly regarding to reduced microbial eradication efficiencies (Bourion and Cerf 1996; Erb et al. 1997; Budhani and Struthers 1998; Skillman et al. 1999; Cowan et al. 2000; Leriche et al. 2003). Various hypotheses have been put forward to explain microbial protection against chemical agents, such as enzyme complementation (Shu et al. 2003) and organized spatial distribution of the cells in the biofilms (Cowan et al. 2000; Leriche et al. 2003). Besides protection against antimicrobial agents, life in multi-species biofilms communities offer beneficial interactions, which include the ability to acquire transmissible genetic elements by conjugation (Ghigo 2001) and coaggregation of cells, which is thought to determine the distribution of bacteria within the biofilm (Tait et al. 2002; Rickard et al. 2003; Sharma et al. 2005a,b).

Phages can effectively infect and lyse cells present in single species biofilms (Hughes et al. 1998; Hanlon et al. 2001; Tait et al. 2002; Sillankorva et al. 2004; Sharma et al. 2005a; Curtin and Donlan 2006; Lu and Collins 2007; Sillankorva et al. 2008c). However, to the authors' knowledge only one phage infection study has been performed on dual species biofilms (Tait et al. 2002).

In the present study, a newly isolated polyvalent *Staphylococcus* phage was combined with a highly efficient *Pseudomonas* T7-like phage (phage ϕIBB-PF7A),

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previously used in *Pseudomonas fluorescens* biofilm control studies (Sillankorva et al. 2008b), to target dual species biofilms of these both hosts. Furthermore, whether the less predominant species present within dual species biofilms could be reached, infected and killed by its specific phage was evaluated. Additionally, the effect of dynamic *vs* static phage infection conditions in the overall removal of biofilm biomass was tested.

Materials and methods

Bacteria, bacteriophage and growth conditions

P. fluorescens PF7 and *Staphylococcus lentus* SL58 were isolated from a dairy plant (Paços de Ferreira, Portugal) after routine equipment cleaning procedures and identified by API strips (API 20NE and API Staph). Both bacteria were grown at 30°C in Tryptic Soy Broth (TSB, Fluka) or in solid TSA medium containing 1.2% w/v Bacto™ agar (Difco). Bacteriophages ϕIBB-PF7A for *P. fluorescens* and ϕIBB-SL58B for *S. lentus* were isolated from raw sewage (wastewater treatment plant, Esposende, Portugal) purified and concentrated using the double soft agar layer (Sambrook and Russell, 2001) where the soft agar top-layer of TSB contained 0.6% Bacto™ agar.

Phage characteristics

S. lentus phage ϕIBB-SL58B and *P. fluorescens* phage ϕIBB-PF7A, belong to the Podoviridae family characterized by phages with an icosahedral head and a short and non-contractile tail (Figure 1A and B). Phage ϕIBB-PF7A has latent, eclipse and rise periods of 15, 10, and 25 min, respectively, and a burst size of about 153 PFU per infected mid-exponential growth phase cell (see Figure 1C). Furthermore, this phage has already been further characterized including the phage host range, structural proteins and genome size (Sillankorva et al. 2008a). The growth of the novel isolated phage ϕIBB-SL58B is characterized by both latent and eclipse periods of 15 min, a rise period of 20 min and a burst size of 507 PFU per infected mid-exponential growth phase cell (Figure 1C). Using a multiplicity of infection of 0.01, it was observed that, at room temperature and under shaker conditions (150 rpm), both phages adsorbed quickly to steady-state growth phase cells of their respective hosts and that after 5 min of inoculation the percentage of free *P. fluorescens* and *S. lentus* detected was approximately 8 and 4 (Figure 1D), respectively.

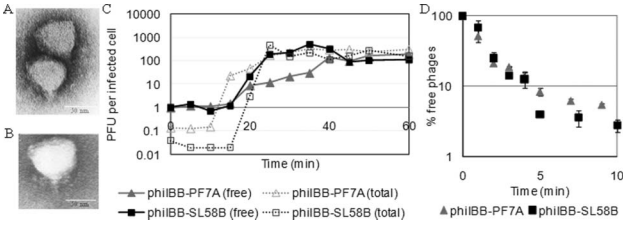


Figure 1. Phage characteristics: (A) EM of phage ϕIBB-PF7A for *P. fluorescens*; (B) EM of phage ϕIBB-SL58B for *S. lentus*; (C) one-step growth curves of phages ϕIBB-PF7A and ϕIBB-SL58B showing the PFU per infected cell in untreated cultures (■) and in chloroform-treated cultures (□); (D) adsorption of phages ϕIBB-PF7A (▲) and ϕIBB-SL58B (■) showing the percentages of free ϕIBB-PF7A and ϕIBB-SL58B phages after infection of steady-state *P. fluorescens* and *S. lentus* at a MOI of 0.01.

Phage titration of stock solution

Bacteriophage titers were performed as described by Adams (1959). Briefly, bacteriophage samples were diluted in SM buffer (5.8 g l^{-1} NaCl, 2 g l^{-1} $MgSO_4 \times 7\ H_2O$, 50 ml l^{-1} 1 M TRIS, pH 7.5). After this, 50 μl of the respective host bacterium, 50 μl of bacteriophage, and 1.5 ml of soft agar top-layer were poured onto two-compartment Petri dishes. The dishes were incubated for 18 h after which the bacteriophage plaque forming units (PFU) were enumerated.

One-step growth curve

One-step growth curves were performed as described by Pajunen et al. (2000) with some modifications. Briefly, 10 ml of a mid-exponential-phase culture were harvested by centrifugation (7000 × *g*, 5 min, 4°C) and resuspended in 5 ml fresh TSB medium to an optical density (OD_{600}) of 1.0 which corresponded to approximately 3.08×10^9 cells ml^{-1} of *S. lentus* and to 1.79×10^9 cells ml^{-1} of *P. fluorescens*. To this suspension, 5 ml of phage solution were added in order to have a multiplicity of infection (MOI) of 0.001. Phages were allowed to adsorb for 5 min at room temperature. The mixture was centrifuged (7000 × *g*, 5 min, 4°C), the pellet resuspended in 10 ml of fresh TSB medium and incubated at room temperature with shaking (150 rpm, Rotamax 120, Heidolph Instruments GmbH & Co., Germany). Two samples were taken every 5 min over a period of 1 h. The first sample was plated immediately without any treatment and the second set of samples was plated after treatment with 1% (v/v) chloroform to release intracellular phages.

Bacteriophage adsorption

Bacteria in the steady-state growth phase were diluted in TSB to an OD_{600} of 1.0. Then, 10 ml of the bacterial

suspension and 10 ml of phage solution were mixed (MOI of 0.01) and incubated at room temperature with shaking (150 rpm). Samples were collected every minute for a total period of 15 min. Samples were immediately chloroform-treated, diluted and plated on TSA plates. After overnight incubation at 30°C phage plaques were counted.

Biofilm formation

Single species biofilms were formed on stainless steel (SS) 1 cm × 1 cm slides, immersed in six-well microplates with 6 ml of TSB medium, for 24 and 72 h under dynamic conditions (shaking) with medium **r**enewal (DR), under dynamic conditions but with no medium **r**enewal (DNR) and under static conditions with medium **r**enewal (SR) as previously described (Sillankorva et al. 2008b). Dual species biofilms were also formed using the procedure described by Sillankorva et al. (2008b) with some modifications. Briefly, the SS slides immersed in 6-well microplates with 6 ml of TSB medium were inoculated with 25 μl (OD_{600nm} of 1.0) of overnight cultures grown at 30°C and 150 rpm of both *S. lentus* and *P. fluorescens*. In single and dual species biofilms, dynamic conditions represented biofilm formation in six-well microplates at 30°C and 100 rpm rotation (Multitron, 2.5 cm amplitude, Infors AG, Bottmingen-Basel, Switzerland) and static conditions represented biofilm growth at 30°C without shaking. In biofilms where the medium was renewed, this medium exchange was carried out every 12 h during the whole duration of the experiment.

Biofilm infection

Biofilms were infected with phages as described previously (Sillankorva et al. 2008b) with some modifications for dual species biofilm experiments. Briefly, SS slides with dual species biofilms were washed by immersion in PBS and placed in new six-well microplates with 3 ml of TSB and 3 ml of phage solution or 3 ml of SM buffer in the case of control experiments. The phage cocktail contained 1.5 ml of 2×10^7 PFU ml^{-1} of *P. fluorescens* phage (ϕIBB-PF7A) and 1.5 ml of 2×10^7 PFU ml^{-1} *S. lentus* phage (ϕIBB-SL58B), so that the titer of each phage in the cocktail was 1×10^7 PFU ml^{-1} while the infection of dual species biofilms using only the *P. fluorescens* phage was performed with 3 ml of phage ϕIBB-PF7A with a titer of 10^7 PFU ml^{-1}. The microplates were incubated at 30°C under the same conditions (static or dynamic) at which the biofilms were formed. Samples were taken after 2 and 4 h for CFU counts and after 4 h for PFU counts.

CFU and PFU counts of attached or planktonic bacteria or phages

The numbers of bacteria present on the SS slides before and after infection of pre-grown biofilms and bacteria released to the planktonic phase after phage infection were enumerated as described by Sillankorva et al. (2008b). Briefly, SS slides with biofilms were washed by immersion in PBS and inserted in 50 ml tubes with 6 ml of saline (0.9% NaCl). The tubes were vigorously vortexed (4 × 30 s) and the SS slides were immediately removed to prevent bacterial reattachment. Afterwards, dilutions were made with saline for CFU counts and plated on TSA Petri dishes. Also, serial dilutions were made with SM buffer, for PFU counts. For counts of the planktonic samples, samples where simply diluted in saline or SM buffer and similarly plated. For *P. fluorescens* cell counts, the selective medium *Pseudomonas* Isolation Agar (PIA) (Sigma-Aldrich, St Louis, MO) was used and the procedure was the same as that adopted for total cell counts. The Petri dishes were incubated at 30°C for 24 h and colonies were counted. Six independent counts were performed for the attached and planktonic CFU and PFU countings.

Electron microscopy

Bacteriophages ϕIBB-SL58B and ϕIBB-PF7A particles were sedimented at 25,000 × *g* for 60 min using a Beckman (Palo Alto, CA) J2-21 centrifuge with a JA 18.1 fixed-angle rotor. Bacteriophages were washed twice in 0.1 M ammonium acetate (pH 7.0), deposited on copper grids provided with carbon-coated Formvar films, stained with 2% potassium phosphotungstate (pH 7.2), and examined in a Philips EM 300 electron microscope (kindly performed by Dr H W Ackermann, Laval University, Quebec, Canada).

Microscopy and image processing

Image acquisition was performed using a Zeiss LSM 5 PASCAL confocal laser scanning microscope (CLSM) (Carl Zeiss MicroImaging GmbH, Jena, Germany). SS slides with biofilms for CLSM were rinsed by immersion in 1 × PBS and immersed into a 2.5% glutaraldehyde solution (4°C, 1 h) for fixation. Afterwards, the SS slides were rinsed with 1 × PBS and stained with 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes, Carlsbad, CA, USA) according to the manufacturer's instructions. The LSM image browser software was used for analysis of biofilm images and z-stacks were acquired from random positions through the biofilm avoiding the edges of the SS slides.

For Field Emission Scanning Electron Microscopy (FESEM), samples were taken before phage and after phage infection for 4 h. The SS slides were rinsed by immersion in PBS before adding 2.5% glutaraldehyde and incubated at 4°C for 1 h. Dehydration was carried out in ethanol series (30%, 50%, 70%, 80%, 90% and absolute ethanol), followed by critical drying (Critical Point Dryer CPD 030). Biofilms were coated with platinum and analyzed by FESEM in a JEOL JSM-6300F (Tokyo, Japan) instrument.

Results

Effect of agitation and nutrient supply on single species biofilm formation

Single species biofilms of *P. fluorescens* and *S. lentus* were formed under three different conditions [**s**tatic with medium **r**enewal (SR), **d**ynamic with medium **r**enewal (DR) and **d**ynamic with **n**o medium **r**enewal (DNR)] and the number of viable cells was determined (Figure 2).

After 24 h, *S. lentus* biofilms revealed similar numbers of viable cells taking account of the formation conditions used. However, 72 h old *S. lentus* biofilms were clearly influenced by nutrient supply since the biofilms grown with medium renewal presented approximately 10 fold more viable cells than biofilms formed in DNR conditions, regardless of whether they were shaken (DR) or static (SR) (Figure 2).

P. fluorescens biofilms formed under static conditions showed significantly more cells than biofilms formed under the two dynamic conditions. In agreement with the *S. lentus* biofilm experiments, *P. fluorescens* biofilms formed under medium with no renewal had fewer cells than those biofilms which experienced regular medium exchange (Figure 2). Compared to *S. lentus, P. fluorescens* cells were less successful in colonizing the metal surface with approximately 10–1000 times fewer cells present (Figure 2).

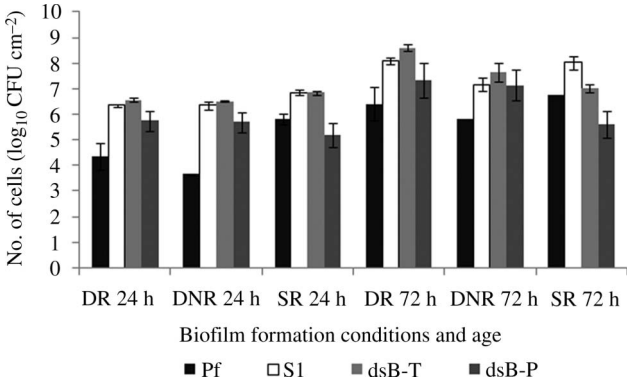


Figure 2. Number of viable cells in *P. fluorescens* (Pf) and *S. lentus* (Sl) biofilms and total cells (dsB-T) and *Pseudomonas* cells (dsB-P) on dual species biofilms formed after 24 and 72 h under dynamic and media renewal conditions (DR), dynamic and media non-renewal conditions (DNR) and under static and media renewal conditions (SR). The error bars represent SDs from three independent experiments performed in duplicate.

Single species biofilms infected with phages

Single species biofilms of *P. fluorescens* and *S. lentus*, formed under the three conditions described above, were infected with their specific phages. The numbers of viable cells and phages remaining in the biofilms and those released to the planktonic phase were enumerated (Figure 3A and B).

In all phage infection experiments, after only 2 h the numbers of cells in the biofilms had decreased considerably, due to lysis caused by their respective phages, and

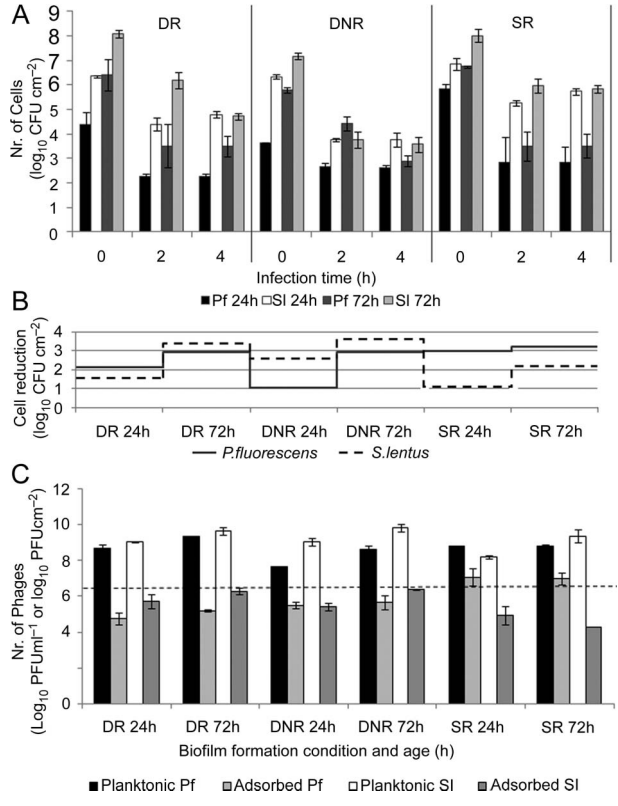


Figure 3. Phage infection of single species *P. fluorescens* (Pf) and *S. lentus* (Sl) biofil-ms. (A) viable cells before and after exposure for 2 and 4 h to the specific phages. Biofilms were formed for 24 and 72 h under dynamic and media renewal conditions (DR), dynamic and media non-renewal conditions (DNR) and under static and media renewal conditions (SR). (B) orders of magnitude of cell reduction observed after treatment for 4 h with phages; (C) number of *P. fluorescens* (Pf) and *S. lentus* (Sl) phages attached to the substrata ($\log_{10}$ PFU cm^{-2}) and on the planktonic phase ($\log_{10}$ PFU ml^{-1}) after treatment for 4 h. The error bars represent SDs from three independent experiments performed in duplicate.

only a small number of *P. fluorescens* and *S. lentus* survivor cells was detected (Figure 3A). In all other phage infection experiments, with the exception of two particular conditions, viz. (i) when 72 h old *P. fluorescens* biofilms formed under DNR, and (ii) when 72 h old *S. lentus* biofilms formed DR conditions were infected, the number of cells was not substantially reduced by prolonging the phage treatment from 2 to 4 h.

Also, after biofilm treatment with phages the overall cell reduction was higher in 72 h than in 24 h old biofilms (Figure 3B) presumably due to a higher initial number of cells present on the SS surfaces. Although this assumption was valid for most of the experiments performed, it is not straighforward. For instance, all 24 h old *S. lentus* biofilms had similar numbers of viable cells, nevertheless, the cell reductions after phage treatment of the DR, DNR, and SR biofilms clearly differed (Figure 3B). Unexpectedly, *S. lentus* biofilms were most reduced under nutrient limited conditions (Figure 3B) while these were the least susceptible to lysis by ϕIBB-SL58B phage under repeated medium renewal and non-shaken conditions (SR). Contrarily to *S. lentus*, phage ϕIBB-PF7A caused the highest *P. fluorescens* cell reduction in 24 h old biofilms formed under SR conditions, which was about two orders of magnitude higher than in SR *S. lentus* biofilms, and approximately 1.5 and 0.5 orders of magnitude higher than after infection of *S. lentus* biofilms under dynamic conditions (DR and DNR) respectively (Figure 3B).

Single species biofilms formed under dynamic and medium renewal (DR) conditions over 72 h were observed using FESEM before and 4 h after infection (Figure 4). *S. lentus* biofilms consisted of thick microcolonies, whereas *P. fluorescens* biofilms showed two distinct cell morphologies, rod and filamentous like, which were attached to the SS slides. After phage treatment, the SS slides had visible amounts of cellular and biofilm matrix debris (Figure 4).

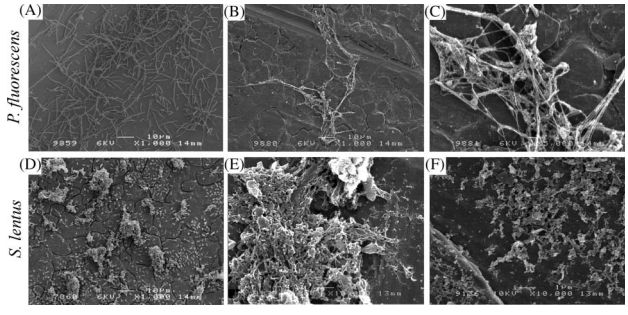


Figure 4. FESEM images of the overall coverage of SS slides with single species biofilms of *P. fluorescens* and *S. lentus* before and after phage treatment. Biofilms were formed under dynamic medium renewal (DR) conditions for 72 h (A, D) and afterwards infected with the respective specific phages ϕIBB-PF7A (B, C) and ϕIBB-SL58B (D, E). Scale bars correspond to 10 μm in figures taken at 1000 × magnification and to 1 μm at 5000 × and 10,000 × magnifications, respectively.

Furthermore, phage infection resulted in a considerably higher number of phages in the planktonic phase as well as a greater number of phages attached to the substratum and the remaining biofilm (Figure 3C). As expected, the higher number of cells in the biofilm resulted in a more efficient adsorption of the released phages, which finally led to more efficient lysis of the biofilm and locally to a higher phage concentration either in the biofilm and substratum or released to the planktonic phase. Static infection conditions were shown to be favorable for the attachment of the *Pseudomonas* phage, while the *Staphylococcus* phage was best retained on the SS when shaking was applied (Figure 3C).

Characterization of dual species biofilms

P. fluorescens and *S. lentus* were used to produce dual species biofilms using the biofilm formation methods DR, DNR and SR described above. Total (dsB-T) and *P. fluorescens* (dsB-P) cells were measured and are presented in Figure 2. CLSM was used to assess the overall coverage of the SS slides by the different biofilms studied (Figure 5).

Dual species biofilms formed over both 24 and 72 h consisted predominantly of *S. lentus* cells and only low numbers of *P. fluorescens* were detected (Figure 2). The total number of cells in 24 h old dual species biofilms was not influenced by the renewal of the medium or by shaking (Figure 2). Furthermore, 24 h biofilms consisted of a sparse single layer of cells with a small number of cell clusters and aggregates (Figure 5). After 72 h, there was an enhancement in the number of viable cells especially under the influence of fluidic and medium renewal conditions (Figure 2). Also, the architecture of the 72 h old biofilms was more developed and they showed a noticeable increase in the size and number of cell clusters, greater coverage of the SS slides and presented thicker biofilms (Figure 5).

Contrary to the single species biofilms, which exhibited the highest cell numbers when formed under SR conditions, the dual species biofilms were inhibited using SR conditions as observed by the reduction in both the total number of cells (dsB-T) and of *P. fluorescens* cells (dsB-P) (Figure 2) which was confirmed by CLSM (Figure 5).

Challenging dual species biofilms with phages

Dual species biofilm control was performed using two strategies: (i) using a cocktail of both *P. fluorescens*

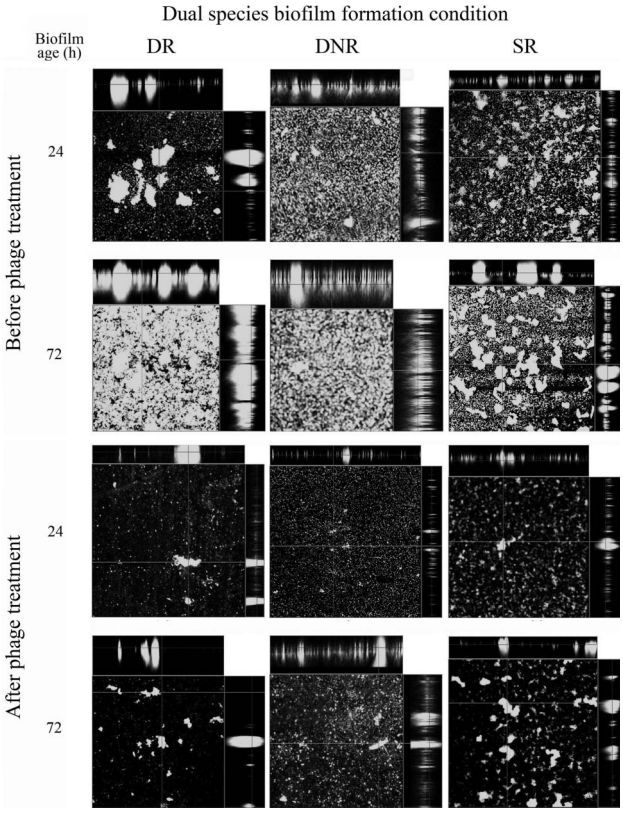


Figure 5. CLSM images (20 × objective) of the overall coverage of SS slides with dual species biofilms before and after phage treatment. Biofilms were formed under dynamic medium renewal (DR), dynamic non-medium renewal (DNR) and static medium renewal (SR) conditions for 24 and 72 h and afterwards infected using a cocktail of phages ϕIBB-PF7A and ϕIBB-SL58B. Biofilms were stained with DAPI after infection for 4 h with the phage cocktail. Orthogonal sections show side views (xz and yz) of the biofilm z-stack images at the selected points.

(ϕIBB-PF7A) and *S. lentus* (ϕIBB-SL58B) phages or (ii) using only the phage for *P. fluorescens* which was less predominant. Phage infection was evaluated by viable counts of the total cells (dsB-T) or *Pseudomonas* numbers (dsB-P) (Figure 6A and B) and also by assessing by the number of phages (Figure 6D).

Challenging dual species biofilms with a phage cocktail

The phage cocktail applied to all dual species biofilms caused a reduction in the biofilms comparative to the controls (biofilms + TSB (1:1 v/v)) (Figure 6A). The cocktail of phages was particularly efficient in reducing 72 h old biofilms formed under dynamic conditions, where the cell concentration was reduced by approximately four orders of magnitude (Figure 6C). Phages were the least efficient in destroying the biofilms under non-shaken (SR) conditions where only a 10 fold decrease after phage treatment for 4 h was observed (Figure 6C). CLSM micrographs confirmed the removal of cells from biofilms formed under shaken conditions and showed that phage treatment was ineffective in biofilms formed under static conditions (Figure 5). The application of phages to the biofilms induced a considerable release of viable cells (approximately of 10^3 CFU ml^{-1}) to the planktonic phase. Both phages, ϕIBB-SL58B and ϕIBB-PF7A respectively, were able to replicate well in the dual species biofilms (Figure 6D). However, the numbers of progeny phages adsorbed to dual species biofilms and to the substrata were lower than observed in single species biofilm infection experiments with each specific phage (compare Figures 3C and 6D). Overall, the phage infection of dual species biofilms followed the kinetics observed with *S. lentus* and its specific phage ϕIBB-SL58B (Figure 3). This was expected since all dual species biofilms were predominantly formed by *S. lentus*.

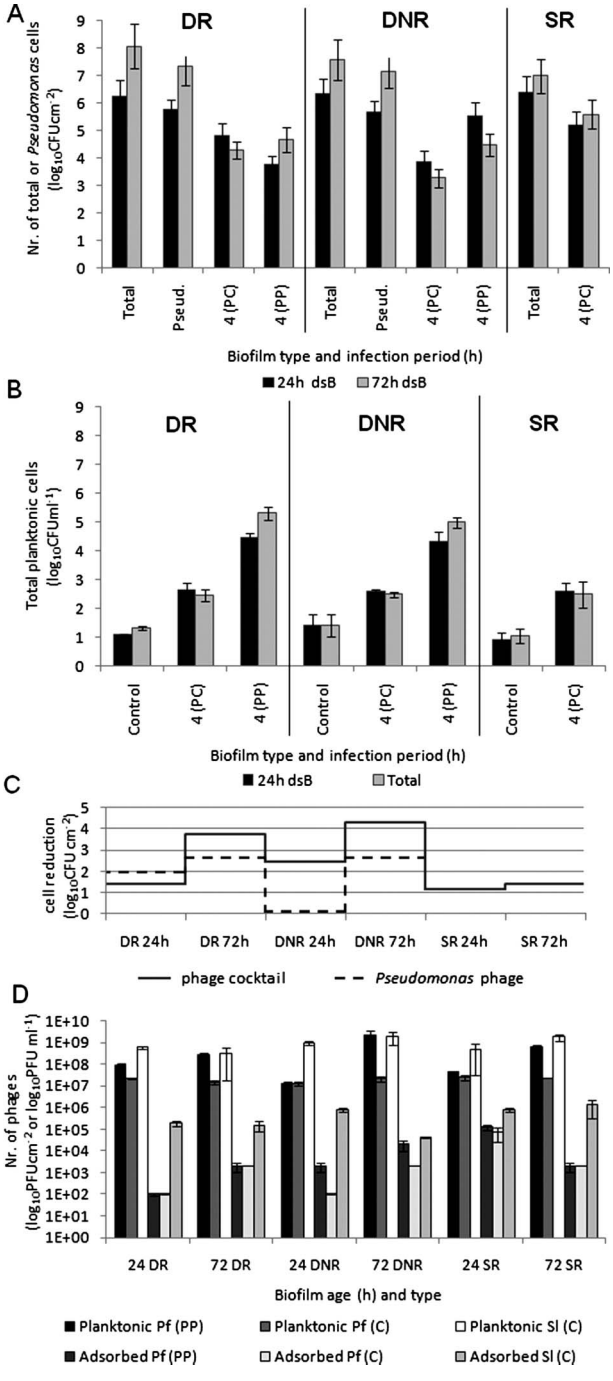
Challenging the less predominant species of dual species biofilms with a specific phage

The second strategy used in this work consisted in exposing dual species biofilms to a single phage specific for the less predominant bacterium present. Dual species biofilms formed under dynamic conditions (DR and DNR) were predominantly formed by *S. lentus* while in static (SR) biofilms the viable cell counts of both *P. fluorescens* and *S. lentus* were similar (Figure 2). Thus, only the two dynamic conditions (DR and DNR) were challenged. The numbers of *P. fluorescens* cells in the dual species DR and DNR biofilms, the total cells released to the planktonic media and the number of phages were enumerated (Figure 6).

After exposure to phage ϕIBB-PF7A the phage successfully reached its host (Figure 6A) and, with the exception of 24 h old DNR biofilms, caused lysis of *P. fluorescens* from all other biofilms as demonstrated from the *P. fluorescens* cell reductions represented in Figure 6C. Furthermore, phages were generally capable of reducing the numbers of viable cells by almost three orders of magnitude, even from 72 h old infected DNR biofilms (Figure 6). Furthermore, the infection of dual species biofilms with the *Pseudomonas* phage alone resulted in a 100-fold increase in the number of cells present in the planktonic phase compared to the biofilm treatments with a cocktail of phages (Figure 6B). Samples taken after 4 h of phage infection from the planktonic phase of DR and DNR infected biofilms revealed the widespread presence of *S. lentus* when analyzed by

light microscopy after Gram staining (data not shown) suggesting that *S. lentus*, the predominant organism in the biofilms had detached.

The data also suggest that the *Pseudomonas* phage can more successfully reach its host when applied alone rather than in a cocktail, since the number of phages adsorbed and in the planktonic phase were greater (Figure 6D) when present alone.



Discussion

In this work, phage control of dual species biofilms of two bacteria that co-exist in dairy plants was studied. To the authors' knowledge, this is the first study where dual species biofilms formed by a Gram-positive (*S. lentus*) and a Gram-negative bacterium (*P. fluorescens*) were subjected to lytic phages. Phage control of such biofilms was investigated by two approaches, with phages specific for either one or both of the hosts, in order to determine the extent of biofilm control in these two different scenarios.

This experimental study provides evidence that a single phage applied to dual species biofilms can effectively reach the host and reduce the cell numbers in the biofilms, but also may cause the release of the non-susceptible species to the planktonic phase. Furthermore, the application of a phage cocktail for each of the hosts present in dual species biofilms decreased not only the cell number in the biofilm, but controlled also the cells which were released from the biofilms into the planktonic phase. Moreover, the conditions under which the phages are applied are important factors to be considered. Accordingly, single species biofilm destruction and cell lysis were efficient when biofilm formation and phage infection were performed under static conditions. However phages were incapable of decreasing the number of bacterial hosts when dual species biofilms were handled in the same way. Thus, without shaking, *S. lentus* and *P. fluorescens* dual species biofilms appeared to be protected from phage infection. The results of this work are in agreement with the work of Tait et al. (2002) suggesting that in a mixed biofilm the presence of a non-susceptible bacterial population within a biofilm can protect phage-susceptible bacteria from

Figure 6. Phage infection of 24 and 72 h-old dual species biofilms (dsB). (A) Number of total cells of *P. fluorescens* present on biofilms after infection for 4 h using a phage cocktail (PC) or the *Pseudomonas* phage (PP); (B) number of total cells present on the planktonic phase after infection for 4 h using a phage cocktail (PC) or the *Pseudomonas* phage (PP); (C) orders of magnitude reduction in the total cells using a phage cocktail (solid line) or reduction of *P. fluorescens* cells using the *Pseudomonas* phage (dashed line) after phage treatment for 4 h; (D) number of *S. lentus* (Sl) and *P. fluorescens* phages (Pf) on the planktonic medium ($\log_{10}$ PFU ml^{-1}) or attached to a SS slide and biofilms ($\log_{10}$ PFU cm^{-2}) after 4 h of phage infection of different dual species biofilms. The horizontal dashed line represents the number of phages applied for the treatment of the biofilms. Phage infected biofilms were formed under dynamic media renewal (DR), dynamic non media renewal (DNR) and static media renewal (SR) conditions. Controls were biofilms exposed for 4 h to a saline-TSB solution. The error bars represent SDs from three independent experiments performed in duplicate.

being attacked by the phage. However, in the present case it cannot be assumed that bacterial protection always takes place as evidenced by the very efficient cell reductions obtained after phage infecting mixed biofilms under dynamic conditions (DR and DNR biofilms) not only with the phage cocktail but also with the *Pseudomonas* phage alone. Furthermore, the less successful decrease under static conditions, after phage exposure for 4 h, may be due to a lower efficiency of the interaction of the phages with their hosts. Another possibility for the low phage infection efficacies observed in static biofilms may be a higher polysaccharide and protein content than in dynamic biofilms. It is known that biofilms formed under distinct hydrodynamic conditions exhibit different amounts of cellular and extracellular polysaccharides and proteins. Simões et al. (2007) showed that biofilms of *P. fluorescens* ATCC 13525^T formed under laminar flow conditions have a much higher content of cellular and extracellular polysaccharides and proteins than turbulent flow-generated biofilms. However, the explanation for the different phage performance detected in shaken and non-shaken biofilms needs further investigation to be proved.

The addition of a single phage or the two-phage cocktail to dynamic dual species biofilms resulted in a 1000 fold reduction in the cell number in 72 h old dual-species biofilms within a short exposure period of 4 h. This efficiency is comparable with other reported studies of biofilms infected with phages over a 24 h period (Hanlon et al. 2001; Tait et al. 2002; Sharma et al. 2005a; Lu and Collins 2007). In the present study, single species biofilms exposed for 2 h to phages caused a major reduction in the biofilm, and prolonging the exposure period to 4 h reduced only insignificantly the remaining biofilm. The results of this work are in agreement with those obtained by Hughes et al. (1998) where the maximum reduction in the viable cells in biofilms was obtained within 2 h or 5 h, depending on the biofilm studied, after which there was no further decrease. The phage exposure period adopted for the dual species biofilm treatments with phages was 4 h and the results obtained clearly indicate that prolonged phage infection periods are not required. Previous experiments with wild and genetically modified T7 phages have revealed that the biofilm cells of different hosts (*P. fluorescens* ATCC 27662 and *P. fluorescens* PF7A, *Enterobacter cloace* NCTC 5920, *Enterobacter agglomerans* Ent and *Escherichia coli* TG1) showed no signs of resistance after 3 and 4 h (Sillankorva et al. 2008b,c), 24 h (Tait et al. 2002) and even after 48 h incubation with the respective phages (Lu and Collins 2007). Nevertheless, resistance acquisition by the hosts will unquestionably appear, especially when long periods of incubation with phages are used, and thus whenever possible the incubation periods should be restricted to as short as possible.

The use of phages to treat and control established single species biofilms of *P. fluorescens* and *S. lentus* indicate the possibility of obtaining efficient reductions in biofilm cell numbers. *P. fluorescens* biofilms were well infected under static conditions while under these conditions *S. lentus* biofilms were inefficiently attacked. It was also noticed that phage treatment of *P. fluorescens* biofilms was less efficient when the biofilms were grown without medium renewal, essentially due to the lower biofilm formation ability of *P. fluorescens* when grown under these conditions. In contrast and despite the similar number of cells in the three distinct *S. lentus* biofilms (DR, DNR, and SR), the *S. lentus* cells were best removed from biofilms formed under nutrient limited conditions. This finding is especially interesting, since the *S. lentus* biofilms were growing in a less favorable environment due to the lack of fresh medium, and especially since it is well known that phage propagation depends on the physiological state of the host which directly reflects the intracellular resources available for phage reproduction (Hadas et al. 1997). The efficiency of biofilm removal was well in accordance with the different burst sizes of the phages used which were approximately 507 PFU per infected cell for the *Staphylococcus* phage ϕIBB-SL58B and about 153 PFU per infected cell for the *Pseudomonas* phage ϕIBB-PF7A respectively.

This study shows the importance of selecting a proper model system for the development of biofilm control studies with phages. Factors such as mixing and the medium supply can influence the infection process. However, they may be optimized to obtain efficient biofilm control. Nevertheless, this investigation proves that both single and dual species biofilms can be efficiently controlled by phages and despite the presence of a non-susceptible host, phages can successfully reach and lyse their target host bacterium. However, for technical applications the effect of the biological control may be enhanced by using other methods along with phages, such as chemical agents to obtain complete biofilm eradication, but this was not part of the present study. The potential of such a combination seems to be attractive, as the results described in this work indicate that even the use of a single phage weakens the biofilm and leads to the release of non-attacked bacterial cells into the medium, where they could be efficiently killed. Also, it seems likely that the number of chemical agents used for biofilm control could be significantly reduced if the biofilms are already partially disintegrated by phages.

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